"IN VIVO AND IN VITRO EVALUATION OF HEPATOPROTECTIVE ACTIVITY, CYTOTOXICITY AND ANTITYPHOIDAL POTENTIAL OF *ENICOSTEMMA* SPECIES FROM MARATHWADA"

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A

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1. A BRIEF RESUME OF PROPOSED WORK / PROBLEM

Medicinal plant sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal arena of rural and tribal lives of India.

Liver diseases are fast being recognized as public health priorities in India. **The burden of liver disease in India is significant because it alone contributed to 18.3% of the two million global liver disease–related deaths in 2015.**

Increasing impact of liver diseases on the country's economy and health care resources, apart from being a cause of premature death and disability. The cultural–lifestyle transition that India is passing through currently with progressive adoption of a western diet, sedentary habits, along with an aura of freedom from long-held taboos around alcohol in the society create grounds for a spectrum of liver diseases in India that shows signs of a rapid switch. This change includes increasing overall importance of alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) as causes of liver disease.

Finding and evaluating the hepatoprotective activity, which is helpful in diagnosis in liver diseases, also work on the cytotoxicity activity and antityphoidal potential activity.

2. INTRODUCTION

Enicostemma axillare belongs to family Gentianaceae. It is a perennial herb found throughout India and is common in coastal areas. The plant is used in folk medicine to treat diabetes mellitus, rheumatisum, abdominal ulcers, hernia, swelling, itching and insect poisoning (Kirtikar and Basu, 1999), anti-inflammatory (Sadique et al., 1987), hypoglycemic (Jyoti, et al ., 2000, Murili, et al., 2002, Jyoti, et al., 2003) and anticancer (Murili, et al., 2002) activities have been reported. These reported activities and many of the ethnobotanical used of the plant related to its hepatoprotective activity. Swertimarin, alkaloids, steroids, triterpenoids, saponins, flavonoids, xanthones, and phenolic acid were isolated from the plant (Kavimani, S. and Manisenthilkumar, 2000). Many such compounds have protective effects due to their pharmacological activities (Wargovich et al, 2001). Liver disease remains one of

the serious health problems. Herbs play a major role in the management of various liver disorders. A number of plants possess hepatoprotective property (Heba et al, 2006). The present study was designed with an aim to assess the hepatoprotective activity of the water extract of arial parts of *Enicostemma axillare*, against paracetamol induced liver damage. **(Gite et al., 2010)**

Enicostema hyssopifolium has been extensive studied, most of it in the last half of the 20th century, and much of it concentrating on *Enicostema hyssopifolium* pharmacological composition, safety, efficacy, and mechanisms of action. A good deal of this research has centered around a screening technique called signal transduction technology. *Enicostema hyssopifolium* is very useful in hepatoprotective action. It improves non-specific immune response. Extracts of *Enicostema hyssopifolium* have been shown to stimulate powerful immune responses in living creatures. The immune response may be specific directed at a microbial invader already present in the body, or generally, strengthening the immune system in preparation against future infections. *Enicostema hyssopifolium* strongly stimulates phagocytosis and the production of specific antibodies. *Enicostema hyssopifolium* is an herb commonly used in China, Srilanka, India and other countries in subtropical and Southeast Asia. Both the fresh and dried leaves, as well as the fresh juice of the whole plant, have been used in a variety of cultures. **(Dr. Aarti Tiwari, 2016)**

The World Health Organization (WHO) estimates that 80% of the people of developing countries rely on traditional medicines, mostly plant-derived drugs, for their primary health needs. Medicinal plants are commonly used in treating and preventing specific ailments and are considered to play a significant role in health care. Use of plants in Traditional medicinal systems is an indispensable source of medicinal preparations. Hundreds of species are recognized as having medicinally value. Indeed, Phytomedicines are beginning to link traditional and modern medicines. **(Jayanthi and Giri, 2016)**

Local name of plant is Nai. The leaves, flowers and root parts of this plant has been traditionally used as a folk remedy for the treatment of Jaundice, blood purifier, lactagogue, in Maharashtra, India, especially by the tribal peoples of Western Ghats. At present, one of the plant-derived medicine approved for use in liver cirrhosis and alcoholic liver diseases is silymarin. There are number of studies which conclude the efficacy of silymarin in these conditions. Silymarin is a mixture of flavonolignans from the fruits of *Silybum marianum* that

has been known since ancient time and recommended in traditional European and Asian medicine mainly for the treatment of liver disorder. Therefore in the present study silymarin was used as positive control to compare the efficacy of *Enicostema axillare* agains*t* CCl4-induced hepatotoxicity. **(Gite et al., 2007).**

Traditional medicine system of India comprised varieties of plants which are playing a significant role in curing diseases from ancient times. Among them, *Enicostemma littorale* blume (*E. littorale*) a perennial herb of the family Gentianaceae is cosmopolitan in occurrence in India. The bittery natured plant acts as a laxative, helps in curing fever, rheumatism, skin diseases, abdominal disorders, snake bite, obesity and helps to regulate blood sugar levels. The plant constituents have been reported for possessing antimicrobial, antioxidant, antiulcer, anti-inflammatory, hypolipidaemic, hepatoprotective and hypoglycemic properties. This review provides a bird's eye view about geographical distribution, physicochemical parameters, phytoconstituents and pharmacological properties of *E. littorale*. **(Rajamani et el., 2013)**

The World Health Organisation **(WHO)** estimated that 80% of the population of developing countries rely on traditional medicine mostly plant drugs, for their primary health care needs Medicinal plants being natural, non-narcotic, having no side elect of ay a range of safe, cost effective, preventive and curative therapies which could be useful in achieving the goal of **"Health for all"** in a coat effective manner. Medicinal plants occupied an important position in the socio-cultural, spiritual and medicinal arena of rural people of India. In recent years interest in medicinal plants has increased considerably world wide. However, it is felt that there seems a very little literature available which addresses to all aspects of medicinal plant species from the point of view of Ayurveda, botany, ecology, chemical and medical science. Medicinal plants based traditional systems of medicines are playing important role in providing health care to large section of population, especially in developing countries. Interest in them and utilization of herbal products produced based on them is increasing in developed countries also. To obtain optimum benefit and to understand the way these systems function, it is necessary to have minimum basic level information on their different aspects. Indian Systems of Medicine are among the well-known global traditional systems of medicine. **(https://www.ncbi.nlm.nih.gov).**

Ayurveda uses the inherent power of natural herbs to bring out wonderful results on the human body. The herbs are natural and 100% safe for human body. A perennial herb

Enicostemma littorale blume (E. littorale) family Gentianaceae is cosmopolitan in occurrence in India. The plant constituents have been reported for possessing antimicrobial, antioxidant, antiulcer, antiinflammatory, hypolipidemic, hepatoprotective and hypoglycemic properties. *E. littorale* is a good source of iron, potassium, sodium, calcium, magnesium, silica, chloride, sulphate, phosphate and vitamins B and C. In recent years, pharmaceutical companies have spent considerable time and money in developing therapeutics based upon natural products extracted from plants. The rising incidence of multidrug resistance amongst pathogenic microbes has further necessitated the need to search for newer antibiotic sources because of its abundant and widespread availability. **(Mishra et al., 2017)**

The plant is native to tropical Africa, Southeast Asia, India and Malaysia; Africa to Lesser Sunda Islands.

Enicostemma littorale Blume and *Enicostema axillare* (Lam.) A. Raynal, Family: Gentianaceae (Gentian Family)

Ayurvedic common names: Marathi : Naai

Maharishi Ayurveda Products, incorrectly refers to *E. littorale* as Indian Gentian; this English common name generally refers to Swertia spp. Whitehead is a common name. The word *Enicostemma* is probably formed from the three words, “en” means inside, “icos” means 20 and “stemma” means wreath or circle due to the many flowers arranged in circles in the leaf axils along the stem. States of Gujarat, Maharashtra, Rajasthan, and Madhya Pradesh are suitable for its cultivation. However, it can also be grown in some areas of Andhra Pradesh, Karnataka and Tamil Nadu having above mentioned climatic conditions.

(Vinotha Sanmugarajah, 2020).

The hepatomodulatory response of ethanol extract of *Enicostemma littorale* Blume was examined in contrast to oxidative stress-induced liver injury by carbon tetrachloride (CCl4) in albino wistar male rats. The rats subjected only CCl4 with olive oil, showed remarkable oxidative stress-induced liver injury. Supplementation of *E. littorale* extract significantly ($P< 0.001$) increased the hepatic reduced glutathione(GSH), glutathione-s-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and vitamin-C (vit.C) in the liver, with a dose-dependent reduction of the thiobarbituric acid reactant substances (TBARS) as evidenced by reduced hepatic lipid peroxidation (LPO)levels, compared with the control animals. The hepatic total cholesterol and triglycerides level were also significantly decreased in the groups receiving *E. littorale* extract, in comparison to controls. Further, the hepatic marker levels-AST, ALT (aspartate and alanine transaminases), ALP (alkaline phosphatase),ACP (acid phosphatase), g-GTP (gamma glutamyl transpeptidase), LDH (lactate dehydrogenase), SDH (sorbitol dehydrogenase), Total bilirubin, total protein and albumin in serum were also restored to normal level dose-dependently after the supplementation of *E. littorale* extract in comparison to respective controls. These biochemical observations were strongly supported by a comparative histoarchitectural examination of liver sections from treated groups, further corroborated the hepatomodulatory role of *E. littole* extract. Silymarin was used as standard drug for comparison with the *E. littorale* extract. In conclusion, these results suggest the hepatomodulation by *E. littorale* Blume against oxidative stress mediated through interference with free radical generation and reduction in fat metabolism.
(Gupta and singh, 2007)

In India, *Enicostemma axillare* is used by the traditional healers for the treatment of liver disorders, which we have scientifically validated in our earlier study in rats. To ascertain and establish the safety for its use in traditional practice, acute and sub-acute toxicity studies of the aqueous extract of *Enicostemma axillare* were carried out as per OCED guidelines. (**Rajasekaran and Arivukkarasu , 2016)**

The green plants provide food, clothing, shelter, and all other needs for human beings (Mathew and Babu 2011).Plant identification is necessary as far as the actual sample of crude drugs is required for the efficacy of the natural medicines. Medicinal plants have been recognized from time immemorial and are also known throughout the world as a rich source

of therapeutic agents in preventing various diseases Medicinal plants play a very active role in traditional medicines to treat various ailments. According to the World Health Organization (WHO 2011), it is estimated that 80% of the whole world's population, especially for millions of vast rural areas of developing countries, people use medicinal plants for the treatment of various types of diseases as a primary health care needs. **(Dhawale et al. 2021).**

3. REVIEW OF LITERATURE AND DEVELOPMENT IN THE SUBJECT:

Gupta and singh (2007) has examined the hepatomodulatory response of ethanol extract of *Enicostemma littorale* Blume in contrast to oxidative stress-induced liver injury by carbon tetrachloride (CCl4) in albino wistar male rats. The rats subjected only CCl4 with olive oil, showed remarkable oxidative stress-induced liver injury. Supplementation of *E. littorale* extract significantly ($P< 0.001$) increased the hepatic reduced glutathione (GSH), glutathione-s-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and vitamin-C (vit. C) in the liver, with a dose-dependent reduction of the thiobarbituric acid reactant substances (TBARS) as evidenced by reduced hepatic lipid peroxidation (LPO)levels, compared with the control animals. The hepatic total cholesterol and triglycerides level were also significantly decreased in the groups receiving *E. littorale* extract, in comparison to controls. Further, the hepatic marker levels-AST, ALT (aspartate and alanine transaminases), ALP (alkaline phosphatase),ACP (acid phosphatase), g-GTP (gamma glutamyl transpeptidase), LDH (lactate dehydrogenase), SDH (sorbitol dehydrogenase), Total bilirubin, total protein and albumin in serum were also restored to normal level dose-dependently after the supplementation of *E. littorale* extract in comparison to respective controls. These biochemical observations were strongly supported by a comparative histoarchitectural examination of liver sections from treated groups, further corroborated the hepatomodulatory role of *E. littole* extract. Silymarin was used as standard drug for comparison with the *E. littorale* extract. hence these results suggest the hepatomodulation by *E. littorale* Blume against oxidative stress mediated through interference with free radical generation and reduction in fat metabolism.

Aarti Tiwari (2016) studied that, the primitive knowledge of plants is important not just for the tribal people, but for the wider world. The use of plants in folk medicine is very prevalent in Central India. During present investigation it was observed that *Enicostema*

hyssopifolium used for various diseases. Considering the medicinal importance of *Enicostema hyssopifolium* a proper policy should be formulated by the govt. to check its exploitation and supply to Ayurvedic medicine industries and the policy framed should be such that it benefit is the tribal and Weaker sections in sum are the other way.

Gite et.al. (2010) performed to assess the hepatoprotective activity in rats against paracetamol as hepatotoxin to prove its claims in folklore practice against liver disorders. In accordance with these results, it may be hypothesized that tannin, saponins and flavonoids, which are present in extracts, could be considered responsible for the hepatoprotective activity. The water extract of aerial parts of *Enicostemma axillare* has shown very significant hepatoprotection against paracetamol -induced hepatotoxicity in albino rats in reducing serum total bilirubin, SALP, SGPT and SGOT levels. It is also found that treatment with water extract of plant has brought down the elevated level of LPO and also significantly enhanced the reduced levels of SOD, CAT, GPX, GST and GSH.

Kavimani and kumar (2000) they reported that *Enicostemma axillare* have many of the ethnobotanical used of the plant related to its hepatoprotective activity. Swertimarin, alkaloids, steroids, triterpenoids, saponins, flavonoids, xanthones, and phenolic acid were isolated from the plant.

Antony et. Al. (2016) studied the flavonoid rich fraction of *E. axillare* can protect the body from oxidative stress may be due to the existence of phytochemicals in the form of flavonoids compounds. These phytochemicals are present in this plant and it contributes to their medicinal properties that can be used in nutraceuticals and pharmaceutical industry. Nevertheless, additional studies are essential to develop technique for the fractionation and identification of most active antioxidant, antibacterial and anticancer molecules and thus can be used in the prevention and treatment of ageing and infective related diseases and can be considered as good source for drug discovery.

Rajasekaran and Arivukkarasu (2016) examined that toxicity study caused no damage to the organs like heart, lungs, liver, spleen and kidney. Abnormalities were also not observed in the organs stomach, testes and ovary of the animals tested at a dose of 200 and 400 mg/kg respectively. The study provided significant data on the sub-acute toxicity profile of AEEA which may be valuable in the clinical study of medicinal herb *Enicostemma axillare*.

Abirami and Gomathinayagam (2011) concluded that promising phytochemicals are widely distributed in *Enicostemma littorale* and it has many pharmacological activities. Animal research has thrown light on anti-inflammatory, analgesic, antimalarial, hepatoprotective and hypoglycemic activities of phytochemicals. Swertiamarin should particularly be screened for large scale clinical trials. Antioxidant and hypoglycemic activities are of special interest.

Vinotha Sanmugarajah (2010) he studied numerous related review provides the *E. littorale* has the number of anti-oxidative phytochemicals, physiochemical properties, chemical constitutions, very low toxicity and is a safe, presence of heavy metals were below the WHO/ FDA permissible limits, and therapeutic pharmacological actions such as anti-diabetic, antioxidant, hypolipidaemic, hepatoprotective, anti-microbial, anti-inflammatory, immunomodulatory, analgesic, anti-arthritic, anti-tumour and etc. This monograph may provide and confirmed the documentary evidence for multiple medicinal and therapeutic uses and pharmacological effects of the *Vellarugu* (*E. littorale*). Longitudinal scientific studies should be carried out in human to prove its clinical standards in the management of the multiple diseases in future.

Vishwakarma and Goyal (2004) studied Phytochemical of this plant showed that it contains triterpeniods, flavonoids, alkaloids and coumarins. Some flavonoids have been reported to inhibit drug metabolism. It is also possible that the active component in the aqueous extract of *E. littorale* may owe its anti-hepatotoxic effect to the inhibition of the biotransformation of CCl4 into the active free radical CCl3. Flavonoids are also known to scavenge free radicals. Based on the results of the present study, it can be suggested that the aqueous extract of *E.littorale* prevents changes in plasma enzyme concentration and other metabolic concentrations as well as diminish the destruction of liver cell architecture initiated by administration of CCl4. Further studies with isolated active principles of the plant may throw more light on the use of *E. littorale* for hepatoprotective activity.

Based upon this results study, it can be concluded that the aqueous extract of *E. littorale* prevent changes in plasma enzyme concentration and other metabolic concentrations as well as diminish the destruction of liver cell architecture initiated by administration of CCl4.

Fadzil et. al. (2021) studied on Swertiamarin for its many health benefits that mainly appear to function through its antioxidant and anti-inflammatory mechanisms. Accumulating evidence provided by various preclinical studies has shown that swertiamarin

can help to treat multiple diseases, based on its analgesic, anti-inflammatory, antiarthritic, hepatoprotective, antidiabetic, antioxidant, antimicrobial, anticancer, neuroprotective and gastroprotective properties.

Swertiamarin can be considered a powerful antioxidant along with multifaceted molecular targets, such as the NF-κB, MAPK/TGF-β1, AMPK, PI3K/Akt, Nrf2-HO-1, and PPARγ signaling pathways, in various pathological conditions. It can modulate multiple signaling pathways, numerous molecular targets, and gene expression. The metabolic processes and pharmacokinetics of swertiamarin in vivo have mostly been investigated in experimental animal models but have not been studied to date in the human body. Therefore, analysis of its toxicities, detailed studies on swertiamarin are required to enhance its bioavailability and to develop novel dosage forms. They also stated that investigations on swertiamarin related to its pharmacokinetics, pharmacodynamics, and clinical and SAR studies and additional parameters will accelerate the development and utilization of swertiamarin as a promising drug candidate in the near future.

Deore et. al. (2008) they were studied in vitro and also conclusion that *Enicostemma* having prominent antioxidant activity due to higher amount of tannis, phenolic and saponin.

4. OBJECTIVES OF THE RESEARCH and PROPOSED HYPOTHESIS

- Collection, identification, authentification and characterization of Enicostemma *sp.*
- To extract the samples in different solvents for partitioning the plant ingredients of polar and non-polar nature.
- To study the *in-vivo* and in-vitro activity of selected plant extracts.
- To study *in-vitro* and in-vitro *hepatoprotective activity,* cytotoxicity, and anti-typhoidal potential of plant extracts.
- To study the *in-vivo hepatoprotective* activity of selected plant extracts by using suitable animal model.
- Statistical study and analysis of all approaches / parameters.

5. MATERIALS AND METHODS

5.1. Plant material and extract preparation

5.1.1. Collection of Plant Material

The native medicinal plant (botanicals) *Enicostemma* species will be selected and collected from Marathwada region to evaluate their, Hepatoprotective activity, Cytotoxicity and antityphoidal efficacy.

5.2. Cleaning and sterilization

5.2.1. Surface Sterilization of Plant Material

The collected plant material will be thoroughly will beheld under the running tap water followed by surface sterilization with 1% Ethanol/H_2O_2 and then will be will beheld with autoclaved distilled water. The surface sterilized plant material will then dried in oven at 40^0 C for 5 days or until they will be dried completely.

5.2.2 Cleaning and sterilization of glasswares and media

All the glass wares required for research work will be kept in cleaning solution for overnight. Cleaning solution will be prepared by dissolving 60g of potassium dichromate ($K_2Cr_2O_7$), 60ml of concentrated sulphuric acid (Conc. H_2SO_4) in one liter of water. Each of these chemicals will be dissolved separately in 500ml of water and finally mixed. All the materials to be used will be will be held thoroughly with sterile distilled water and then dried in hot air oven at 160^0C for 60 min.

The cleaned and dried petriplates will be wrapped with paper and sterilized in autoclave. Test tubes and flasks having culture media will be plugged with non-absorbent cotton. These are covered with paper and further autoclaved at 121^0C at 15 PSI for 20min. The nichrome wire loop, blades, scalpel, forceps will also be dipped in ethanol and then sterilized by heating on the flame.

5.3. Test microorganisms and inoculum preparation

The common typhoid pathogen *Salmonella typhimurium* was brought from Microbial Type Culture Collection and Gene Bank, Chandigarh.

5.4. Organic extracts preparation and their hepatoprotective, cytotoxicity, antityphoidal potential screening

Different organic solvents such as water, ethanol chloroform and hexane will be screened to work out the best extractant. The organic extracts will be prepared by using solvent-solvent extraction of the aqueous extract. The aqueous extract will be prepared by suspending powdered plant material (17.5 g) in sterile distilled water (100 mL) and keeping it in hot water bath at 40 °C for 20 min. Hundred milliliter of filtered aqueous extract will be

then shaken vigorously in a separating funnel with an equal volume of a particular organic solvent, for three independent times. The pooled organic layers will be concentrated in a rotavapor at 45 °C (under vacuum). The leftover material thus obtained will be dissolved in diluted Dimethyl Sulfoxide (DMSO) [(30% (v/v)], which will then be tested for their antimicrobial activity against the reference strains using following technique.

5.4.1. **Disc Diffusion Assay**

5.4.2. Evaluation of hepatoprotective, cytotoxicity, antityphoidal potential

5.4.3. Minimum inhibitory concentration (MIC) studies

The test preparations (ethanol extract, ethyl acetate extract, flavonoids, cardiac glycosides) will be studied for their MIC against the reference microbial strains (sensitive to the respective preparations in Agar Well Diffusion) by Agar dilution method, as per the protocol followed in Sood et al., (2020) using various concentrations prepared from their respective stock solutions. Hundred microliters (100 µL) of the activated test organism (adjusted to 0.5 McFarland standards) will be swabbed onto the solidified plates (containing different concentrations of a test preparation) and incubated at 25 °C for 18–24 h. The lowest concentration of the test preparations which inhibited the microbial growth will be taken as their MIC. The values will be compared with that of the standard antibiotics.

5.5. Qualitative and quantitative phytochemical analysis

5.5.1. Phytochemical Tests

Fresh stock solution of each extract will be prepared for each experiment. To prepare 100 mg/ml extract, 1g of either of the extract will be dissolved in 10 ml of distilled water, shaken vigorously to obtain a homogenous solution except for the glycoside test, for which 100 mg extract will be directly used. The procedures will be used earlierly described by Harborne (1973) and Sofowara (1993).

5.6 IN VITRO ANTIOXIDANT ACTIVITY

5.6.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

Free radical scavenging activity of *Enicostemma* pecies will be determined by using DPPH assay (Wang et al. 2008). The reaction mixture containing a mild series (10–100 gm/ml) of plant phytochemicals will be incubated in MeOH (3.0 ml; 0.3 mM) with DPPH solution. The ingredients will be mixed vigorously and allowed to stand for 30 minutes at room temperature. The absorbance will be measured at 517 nm. The lower absorbance

indicates high DPPH scavenging activity. Ascorbic acid will be used as the reference antioxidant. The percent inhibition of DPPH will be calculated using the formula, [(C–T/C)] x 100, where C is absorbance of the control and T for the test samples.

5.6.2 Hydroxyl Radical Assay

The hydroxyl radical scavenging activity of *Enicostemma* species will be determined by Yang and Guo, 2001. The reaction mixture containing dilution series from 10 to 100 lg/ml of *Enicostemma* species will be incubated with deoxyribose (3.75 mM), H_2O_{23} (1 mM), $FeCl_3$ (100 lM) in phosphate buffer (pH 7.4). The reaction is terminated by thiobarbituric acid (1 ml; 1 % w/v) and trichloroacetic acid (1 ml; 2 % w/v) and then heated in boiling water bath for 15 min. The pink chromogen formed eventually resulted in the formation of thiobarbituric acid reactive substances (TBARS). The content will be cooled and absorbance of mixture will be measured at 535 nm against blank. Tocoferol will be used as the reference antioxidant. The percent inhibition of hydroxyl radical generation will be calculated using the formula, [(C–T/C)] x 100, where C is absorbance of the control and T for the test samples.

5.7. IN VIVO ANTITYPHOIDAL ACTIVITY OF EXTRACTS IN MOUSE MODEL

From the results of the in vitro antioxidant and antityphoidal assay extracts with significant activity will be considered for in vivo testing against (Swiss albino mouse model).

5.8 Experimental Animals and Ethical Considerations

Swiss albino rats of both sexes weighing 30-35 grams will be procured from the National Institute of Virology, Pune.

5.9 Acute Oral Toxicity Study

Acute oral toxicity study of the will be carried out using Swiss albino mice of both sexes. Lorke's method.

5.10 Selection and Preparation of Dose for Pharmacological Screening

The water ethanol, chloroform and hexane extracts of *Enicostemma* species will be suspended in 0.3 percent CMC (*Carboxy Methyl Cellulose*) solution to prepare two dose levels, 2000 and 400mg/kg body weight of the animals.

5.11 PHYSICAL PARAMETERS

5.11.1 Determination of liver weight

The animals will be sacrificed and the livers will be isolated and will beheld with saline and weight determined using electronic weighing balances. The weight of the liver will be expressed in terms of its body weight (Ghosh et al., 2007).

5.11.2 Determination of Liver Volume

After the weight, livers will be released individually into a measuring cylinder with a fixed volume of distilled water or saline and the displaced volume will be recorded. (Ghosh et al., 2007)

5.12 BIOCHEMICAL PARAMETERS

The biochemical parameters will be estimated according to the standard procedure prescribed by the Manufacturing Instruction Manual given in the kit. (Coral Clinical Systems) using Semi Autoanalyzer

5.12.1 Estimation of Serum SGPT

5.12.2 Estimation or Serum SGOT

5.13.3 Estimation of Serum Alkaline Phosphatase (ALP)

5.13.4 Estimation of Serum Bilirubin

5.13.5 Estimation of Serum Cholesterol

5.13.6 Estimation of Serum Total Proteins

5.14 ANTIOXIDANT PARAMETERS

5.14.1 Estimation of Catalase

5.14.2 Superoxide dismutase

5.15 HISTOPATHOLOGICAL STUDIES

5.15.1 Processing of isolated liver

The animals will be sacrificed and the livers of each animal will be separated and cut into small pieces, preserved and fixed in 10% formalin for two days. The liver piece will be then will be held in running water for about 12 hours to remove the formalin and then

dehydrated with isopropyl alcohol of increased potency (70%, 80% and 90%) for 12 hours. Dehydration is then performed using absolute alcohol with about three changes for 12 hours. The dehydration will be performed by discarding all traces of water. Further alcohol will be removed by using chloroform and chloroform removed by paraffin infiltration. The clearing will be completed by using chloroform with two changes for 15 to 20 minutes each. After paraffin infiltration the liver pieces will be placed to automatic tissue processing unit.

5.15.2 Embedding in paraffin vacuum

The hard paraffin will be melted and hot paraffin will be poured into L-shaped blocks. The pieces of liver will be then quickly dipped into the melted paraline and allowed to cool.

5.15.3 Sectioning

The blocks will be extruded using a microtome to obtain sections of 5 thicknesses. The sections will be taken on a micro slide on which egg albumin will be applied. Sections will be allowed to stay in the oven at 60°C for 1 hour. Paraffin melts and denatures egg albumin, which fixes the tissue to slide.

5.15.4. Staining

Eosin is an acid stain, so it causes pink spots on all the cell components that are in the basic form, i.e. the cytoplasm. Hematoxylin, a basic stain that causes a blue stain on all acidic cell components, namely: DNA in the nucleus.

5.16 STATISTICAL ANALYSIS

The results will be reported as mean ± standard error (SE), and the experiments will be replicated three times (n=3).

6. IMPORTANCE OF THE STUDY

- **International status**

The World Health Organization (WHO) estimated that 80% of the population of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contain at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants.

Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic, having no side-effects, easily available at affordable prices and sometime the only source of health care available to the poor.

Recently, WHO (World Health Organization) estimated that 80 percent of people worldwide rely on herbal medicines for some aspect of their primary health care needs.

According to WHO, around 21,000 plant species have the potential for being used as medicinal plants.

- **National status**

In India tribal people are often called "Adivasis" and the government recognizes them as scheduled tribes (STs). Scheduled Tribes (STs) and also Scheduled Castes (SCs) are the disadvantaged sections of the society due to socio-economic exploitation and isolation since times immemorial.

Medicinal plant sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal arena of rural and tribal lives of India. Our per capita annual consumption of drugs of Rs.125 is the lowest in the world mainly because medicinal plants constitute the principal health care resources for the majority of population.

According to an all India ethnobiological survey carried out by the Ministry of Environment and Forests, Government of India, there are over 8000 species of plants being used by the people of India.

Liver diseases are fast being recognized as public health priorities in India. The burden of liver disease in India is significant because it alone contributed to **18.3% of the two million global liver disease–related deaths in 2015.**

Despite this, a fair body of evidence is available that suggests the increasing impact of liver diseases on the country's economy and health care resources, apart from being a cause of premature death and disability. The cultural–lifestyle transition that India is passing through currently with progressive adoption of a western diet, sedentary habits, along with an aura of freedom from long-held taboos around alcohol in the society create grounds for a spectrum of liver diseases in India that shows signs of a rapid switch.

This change includes increasing overall importance of alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) as causes of liver disease over and above viral causative factors.(https://www.censusindia2011.com) (https://cdnbbsr.s3waas.gov.in)

- **Regional status:**

Maharashtra is one of the ten states which have Fifth Schedule areas. With more than ten million tribal persons in the state. The Fifth Schedule areas in Maharashtra cover parts of Marathwada region also.

In Maharashtra total tribal population is 9.35 %

In Marathwada region total tribal population is 3.98 %

In Nanded district total tribal population is 8.39 %

(https://www.censusindia2011.com) (https://cdnbbsr.s3waas.gov.in)

Tribal people are loved to live in or near to the forest area. Hence for a long time they know the different forest species viz. animal and plants. Now focusing to the plant species tribals are much more having knowledge.

Marathwada is well known as drought prone region and most of the people do farming. Specially tribal survive on different sources which are originated from forest areas, because they have been living in remote area, they can't access easily or connect to the main city hence they depend on forest medicinal plants and hence they use very effectively medicinal plants to treat various diseases.

- **Scope of study**

- Treatment with *Enicostemma* spe. is considered very safe as there is no or minimal side effects.
- These studies are in sync with nature, the golden fact is that, use of herbal treatments is independent of any age groups and the sexes.

- *Enicostemma* are considered as a rich resource of ingredients which can be used in drug development either pharmacopeial, non-pharmacopeial or synthetic drugs.

As our lifestyle is now getting techno-savvy, we are moving away from nature. While we cannot escape from nature because we are part of nature. As herbs are natural products, they are free from side effects, they are comparatively safe, eco-friendly and locally available. There is a need to promote them to save the human lives.

7. PROPOSED WORK PLAN – WITH A TENTATIVE CHAPTER SCHEME

8. YEAR-WISE PLAN OF WORK and TARGET TO BE ACHIEVED

1st Year:-

1. Review of Literature.
2. Reference of work.
3. Collection of *Enicostemma* plant species

2nd Year:-

1. Characterization of phytochemical constituents from the *Enicostemma*
2. In vivo Antityphoidal activity hepatoprotective activity and cytotoxicity
3. Statistical study of all approaches.

3rd Year:-Completion of research work.

Compilation

1. Submission of thesis.

9. EXPECTED OUTCOME

Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for various diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments.

Nowadays, people are focused on herbal medicine. They are blindly using medicinal plants to chronic diseases by listening to the Ram Dev Baba and reading the medicinal values of medicinal plants on multimedia. Every plant has hundreds of phytoconstituents which are harmful to the body and may cause hepatotoxicity and hence research should be carried out on the Oral Toxicity of these selected plant extracts.

It will be more beneficial to the society if such herbal remedy found to cure liver diseases and will be helpful to avoid hepatotoxicity if any by crud extracts and other side effects by allopathic treatment.

10. REFERENCES:

1. **Gite V.N., Pokharkar R.D., Chopade V.V. , Takate S.B,(2007)** Hepato-protective activity of *Enicostemmaaxillare*inparacetamol induced hepato-toxicity in albino rats. *Pharmacologyonline 1: 25-30 (2007)*
2. **Dr. Arti Tiwari, (2016),** Economic importance and market value of *EnicostemmaHyssopifolium*(willd.) *International Journal of Advanced Science and Research, verdwww.allsciencejournal.comVolume 1; Issue 8; August 2016; Page No. 12-15.*
3. **Gupta RS, Singh D. (2007)** Hepatomodulatory role of *Enicostemma littorale* Blume against oxidative stress induced liver injury in rats. Afr J Agri Res. 2007; 2:131-138.
4. **Rajasekaran. A, R Arivukkarasu and S Murugesh, (2010)** Hepatoprotective Effect of *Adenema hyssopifolium G.Don* (Gentianaceae) in Carbon Tetrachloride-InducedHepatotoxicity in Rats, *Tropical Journal of Pharmaceutical Research, 2010 ;*

9 :157-163.

5. **Sharada L Derore, S SK hadabadi, Lalita Bhagure and D S Ghorpade,(2008)** *In vitro* antimicrobial and antioxidant studies on Enicostemma littorale (Lam.) Raynal.Leaves, *Natural Product Radiance, 2008 ; 7:409-412.*
6. **S. Jayanthi and R. Sagaya Giri, (2016)** identification of phytochemicals and anti-pyretic activity of *Enicostema axillare* Lam. ,*World Journal of Science and Research 1(1): 26-29 (2016).*
7. **Dr. Nidhi Mishra, Dr. Kamini Kaushal, Dr. Rajesh Chandra Mishra and Dr. Ashwini Kumar Sharma ,(2017)** An ayurvedic herb: Enicostemma littorale blume- A review article , *Journal of Medicinal Plants Studies 2017; 5(1): 78-82*
8. **Abirami and Gomathinayagam,(2011)** a review on enicostemma littorale , *Pharmacologyonline 1: 75-83 (2011)*
9. **Aiyalu Rajasekaran and Ramasamy Arivukkarasu,(2016)** Acute and Sub-acute Toxicity Study of Aqueous Extracts of *Enicostemma axillare* (Lam.) Raynal in Animal models , *Pharmacognosy Journal, Vol 8, Issue 1, Jan-Feb, 2016*
10. **S. L. Vishwakarma, R. K. Goyal**, **(2004)** Hepatoprotective activity of *Enicostemma littorale* in CCl4-induced liver damage, *journal of natural remedies , Vol 4/2 (2004) 120 – 126*
11. **Antony Rose Immaculate C, Umarani V., Sankaranarayanan**, **(2016)** Antioxidant, antibacterial and cytotoxicity studies from flavonoid rich fraction of *Enicostemma axillare* (LAM.) raynal leaves, *African Journal of Pharmacy and Pharmacology ,Vol. 10(43), pp. 916-925, 22 November, 2016*
12. **Vinotha Sanmugarajah**,**(2020**) a review on therapeutic and pharmacognostic properties of *vellarugu* (*enicostemma littorale* blume) , *International Journal of Ayurveda and Pharma Research , IJAPR | April 2020 | Vol 8 | Issue 4*
13. **kanchan sandeep dhawle, umesh pravin dhuldhaj, ramjan mahamuddin mulani, (2021)** Pharmacognostic studies and anatomical peculiarities in medicinal plant *Enicostemma axillare, asian journal of ethnobiology, Volume 4, Number 1, May 2021 E-ISSN: 2580-4537 Pages: 23-30*
14. **Rajamani Saranya1, Thirunavukkarasu Thirumalai, Munisami Hemalatha, Ranganathan Balaji.,(2013)** Pharmacognosy of Enicostemma littorale: A review , *Asian Pac J Trop Biomed 2013; 3(1): 79-84*

15. **Fadzil1 et. al.,(2021)** Chemistry, Pharmacology and Therapeutic Potential of Swertiamarin-A Promising Natural Lead for New Drug Discovery and Development, *Drug Design, Development and Therapy 2021:15*
16. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2816487/

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